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Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment

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Abstract

Objectives: The aim of the study was to verify (i) if crevicular fluid defence variables reflect the changes after surgical periodontal treatment and (ii) if they are in correspondence with changes of these variables in the unstimulated and stimulated whole saliva.

Material and Methods: For 12 male and 13 female volunteers with chronic periodontitis lactoferrin concentration as well as the lysozyme and peroxidase activities were determined in crevicular fluid as well as in unstimulated and stimulated saliva before and 14 days after surgical periodontal treatment by a minimal invasive flap technique.

Results: The lactoferrin concentrations decreased significantly in the crevicular fluid eluting solution from 1.63 to 1.23 mg/l reflecting a decrease in the total amount collected, in unstimulated saliva from 10.54 to 8.96 mg/l, and in stimulated saliva from 9.00 to 7.11 mg/l after treatment. No significant change could be found for lysozyme. Peroxidase activity was significantly reduced from 269.06 to 186.15 U/l only in the crevicular fluid.

Conclusion: The results of this study suggest that (i) the defence factor lactoferrin is suitable for monitoring of periodontal treatment results and (ii) changes of the lactoferrin concentration in crevicular fluid are related with significant changes in unstimulated and stimulated saliva.

Key words: crevicular fluid; lactoferrin; lysozyme; periodontitis; peroxidase; saliva

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Crevicular fluid variables give a site-specific reflection of the microbial infection or the degree of inflammation, respectively, and are partly used for periodontal diagnosis and follow-up (Listgarten 1992, Adonogianaki et al. 1996, Buchmann et al. 2002, Jin et al. 2002). Salivary variables cannot give a site-specific information but may be hopeful candidates for the whole mouth follow-up (for review see Kaufman & Lamster 2000). Several variables of the saliva could be promising to be sensitive enough, for example, higher levels of matrix metalloproteinases (MMPs) could be found not only in the gingival crevicular fluid of periodontitis patients, but also MMP-8 was elevated in the whole stimulated saliva of patients with chronic periodontitis (Ingman et al. 1996). Variables of the local defence are of interest regarding periodontitis. The iron-binding glycoprotein lactoferrin contributes to the salivary defence against *Actinobacillus actionomycetemcomitans* and a negative correlation between salivary lactoferrin and the number of subgingival *A. actinomycetemcomitans* could be found (Groenink et al. 1999). Lactoferrin may either kill *A. actinomycetemcomitans* or interfere with its binding to host cells (Fine & Furgang 2002). Lactoferrin can be found in saliva and crevicular fluid (Tenovuo et al. 1991) and acts bacteriostatically and bactericidally (Friedman et al. 1983, Kalmar & Arnold 1988). In the crevicular fluid significant higher levels of lactoferrin have been found at periodontitis sites in comparison to healthy sites (Adonogianaki et al. 1993). Lysozyme is also found in crevicular fluid and saliva (Tenovuo et al. 1987) and acts against gram-positive and gram-negative bacteria (Schmidt 1982). Syrjänen et al. (1989) found decreased concentrations of lysozyme in saliva of periodontitis patients but

elevated levels in the crevicular fluid. Peroxidase activity of the crevicular fluid derives from blood leucocyte myeloperoxidase, whereas in the saliva peroxidase activity is a mixture of salivary gland peroxidase and to a smaller extent of myeloperoxidase (Grisham & Ryan 1990, Tenovuo et al. 1991). Elevated levels of peroxidase activity in saliva are described for gingivitis and periodontitis patients (Basu et al. 1982, Jentsch & Göcke 1995). Periopathogenic bacteria give rise to the degranulation of leucocytes resulting in the delivery of myeloperoxidase (Courtois et al. 1990). The peroxidase activity in crevicular fluid is enhanced in case of gingivitis and periodontitis (Cao & Smith 1989, Karhuvaara et al. 1990).

The aim of this study was to follow-up periodontal treatment by the same salivary and crevicular fluid variables. It should be verified, if (i) crevicular fluid defence variables reflect the changes after surgical periodontal treatment and (ii) if the same salivary variables reflect the crevicular fluid results in an appropriate manner.

Methods

Twenty-five patients (12 males and 13 females) with chronic periodontitis (mean age: 48.0 ± 9.6 years) gave their written informed consent and participated in the study. The sample size was fixed after model calculations for lactoferrin, lysozyme, and peroxidase activities using $\alpha = 5\%$ for the level of significance and a test power of 80% (Jones & Payne 1997). The criteria for inclusion were: >3 mm pocket depth after initial therapy, no antibiotic therapy in the last 6 months, more than 20 natural teeth, an approximal plaque index (API, Lange 1986) $<30\%$ after initial therapy, sites for periodontal treatment were positive for bleeding on probing. A minimal invasive flap technique followed by scaling and root planing was performed in two quadrants under local anesthesia (one in the maxilla, one in the mandibula of the same side). Prior to treatment as well as after 14 days crevicular fluid was collected from proximal sites at frontal teeth. The deepest site per tooth was used. Periopaper (Pro Flow^{TM}, Amityville, NY, USA) was introduced into the sulcus for 2 min under relative dryness. Preliminary studies in our laboratory had revealed that it is more safe to collect crevicular fluid for 2 min to have an amount sufficient for biochemical analysis in the case of lack of inflammation. The stripes then were stored at -18°C. Six separate unpooled periopaper strips per patient and appointment were collected and eluted with 100 μl of 0.5 M NaCl each after thawing and used for biochemical analysis. Furthermore, at the same day before treatment and after 14 days unstimulated whole saliva was collected by the flow method for 15 min, and paraffin wax-stimulated whole saliva was collected for 5 min under standardized conditions. Salivary samples were centrifuged at $3000 \times g$ and 2–4°C for 15 min before storing the supernatant at -18°C.

The biochemical analysis of the activities of lactoferrin, lysozyme, and peroxidase was done after six to ten weeks of sample storing. Lactoferrin was analysed using a heterogenous enzyme immunoassay (Tessenow & Krüger 1986). A peroxidase-labeled antibody was added and the extinction measured with *o*-phenylenediamine as the substrate at a wavelength of 405 nm. Lysozyme was analysed using the lysoplate method (Osserman and Lawlor 1966). Plates with agarose gel supplemented with *Micrococcus luteus* (Boehringer-Mannheim, Mannheim, Germany) were inoculated with the samples and incubated. After 24 h the diameter of the lysed areas were measured. Hen egg white lysozyme (HEWL, Boehringer-Mannheim, Mannheim, Germany) was used as a standard. Thus, lysozyme activity is expressed here relative to the activity of HEWL of the indicated concentration. The determination of the peroxidase activity was performed according to Mansson-Rahemtulla et al. (1986). 5,5′-Dithiobis-2-nitrobenzoic acid in phosphate buffer pH 5.6, reduced to nitrobenzoic acid by mercaptoethanol, was used as substrate, potassium thiocyanate served as co-substrate. The spectrophotometric analysis was performed at a wavelength of 412 nm.

The statistical analysis was performed after testing for normal distribution by the Kolmogorov–Smirnov test. Accordingly, the paired *t*-test or the Wilcoxon test, respectively, was selected. A level of $\alpha \leqslant 0.05$ was considered to be significant.

Results

The clinical indices at baseline are given in Table 1. The mean pocket depths of the treatment area were 4.8 ± 1.9 mm before treatment. The proximal sutures were removed 7 days after the treatment. The results for the analysed variables in the crevicular fluid and the saliva are given in Table 2. Significant changes occurred at the studied variables of the crevicular fluid. In the eluting solutions, the mean concentration of lactoferrin and the mean peroxidase activity were significantly reduced from 1.63 to 1.23 mg/l and from 269.06 to 186.15 U/l, respectively ($p = 0.004$ and 0.001, respectively). The change in lysozyme activity did not reach the level of significance but it was also reduced from 2.56 to 2.23 mg/l after periodontal therapy. In unstimulated and stimulated whole saliva a decrease occurred at the concentration of lactoferrin and peroxidase activities. The decrease in the concentration of lactoferrin in the unstimulated saliva from 10.54 to 8.96 mg/l and in the stimulated saliva from 9.00 to 7.11 mg/l was significant ($p = 0.004$ and 0.007, respectively). No significant changes could be found for lysozyme and peroxidase activities in the unstimulated and stimulated saliva.

Table 1. Clinical indices at baseline

Variable	
no. of volunteers	25
mean age (years)	48.0 ± 9.6
API (%)	<30
tooth mobility	0 and 1
BOP of the study area	positive
mean probing depth of the study area (mm)	4.8 ± 1.9
attachment loss	35–45%

Discussion

There is evidence that several crevicular fluid variables reflect changes due to periodontal treatment or drugs (Mailhot et al. 1998, Doungudomdacha et al. 2001). A relationship between the occurrence of subgingival *A. actinomycetemcomitans* and the lactoferrin concentration of the saliva could be demonstrated (Groenink et al. 1999). In this study, we wanted to check if there is a coincidence between changes in the crevicular fluid and in the saliva after periodontal treatment. The variables lysozyme, lactoferrin, and peroxidase were studied in the crevicular fluid

Table 2. Lactoferrin, lysozyme, and peroxidase in the saliva and crevicular fluid before and after periodontal therapy

Variable	Before therapy			After therapy			Paired *t*-test
	n	*x*	*s*	*n*	*x*	*s*	*p* =
Crevicular fluid[*]							
lactoferrin (mg/l)	50	1.63	0.53	50	1.23	0.59	0.004
lysozyme (mg/l)	50	2.56	2.07	50	2.23	2.23	0.519
peroxidase (U/l)	50	269.06	137.15	50	186.15	97.30	0.001
Unstimulated saliva							
lactoferrin (mg/l)	25	10.54	4.58	25	8.96	4.22	0.004
lysozyme (mg/l)	25	12.01	20.27	25	16.31	51.67	0.696 (W)
peroxidase (U/l)	25	647.39	437.48	25	677.34	398.28	0.669
Stimulated saliva							
lactoferrin (mg/l)	25	9.00	4.19	25	7.11	3.12	0.007
lysozyme (mg/l)	25	2.67	3.64	25	3.36	5.66	0.968 (W)
peroxidase (U/l)	25	675.10	325.03	25	655.40	362.08	0.784

[*]Concentration in eluting solution. W – Wilcoxon test.

after collection for 2 min as well as in the unstimulated and stimulated saliva. No studies regarding this topic could be found in the literature.

Data on gingival crevicular fluid may be analysed in terms of total amount per standardized collection interval, or in terms of concentration in the crevicular fluid (Lamster et al. 1986, Lamster 1997). Those authors suggested that the total amount of a component is more appropriate for studies in the crevicular fluid. Since here all strips were eluted with the same volume of sodium chloride solution, the total amount of the component is reflected by the concentration or activity. No further data transformation has been done. The chosen time interval of 14 days after treatment for repeating the collection of crevicular fluid and saliva is plausible due to the fact that immediate changes of wound healing within the first 7 days, for example, different phases of inflammation caused by the wound itself, are excluded. Longer time intervals could be influenced again by external factors. In the case of this study it was interesting, if there is a biochemical measurable marker of body fluids that reflect improvement of inflammation due to infection.

Lysozyme proved not to be a suitable marker to follow up the treatment in this study. No significant changes were observed in the crevicular fluid nor in the unstimulated or stimulated saliva. Lysozyme is known as a variable lacking normal distribution in the saliva (Tenovuo 1989). Also in the crevicular fluid, no normal distribution of the lysozyme activity was found. The peroxidase activity was significantly reduced in the crevicular fluid but no significant reduction of the peroxidase activity occurred in the unstimulated or stimulated saliva. Lactoferrin gave significant changes in the crevicular fluid as well as in the unstimulated and stimulated whole saliva. A significant decrease as in the crevicular fluid after the surgical periodontal treatment was observed in both salivas. Higher lactoferrin concentrations of the parotid and whole saliva were also found in localized aggressive periodontitis patients (Fine et al. 2002). Thus, our results are in favour of an improved periodontal condition after treatment. The results for lactoferrin in the crevicular fluid of our study are not in coincidence with the lactoferrin concentration in the crevicular fluid also determined with an ELISA test by Miyasaki et al. (1998). Those results were 68.2 ± 108.7 mg/l with a remarkable coefficient of variation of 159.4% contrary to our results with 32.5% before and 48% after treatment.

It may be concluded from this study that lactoferrin in crevicular fluid as well as in unstimulated or in stimulated saliva might be a suitable marker for monitoring of treatment effects in periodontal disease. This is in coincidence with findings by Tsai et al. (1998), where lactoferrin of the gingival crevicular fluid was considered as a more sensitive marker of periodontal pathology than traditional clinical indices.

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